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Short title/Authors running head: GAD antibody measurement using  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) maintains assay sensitivity  
• R. C. Wyatt et al.

## The first 142 amino acids of glutamate decarboxylase do not contribute to epitopes recognized by autoantibodies associated with Type 1 diabetes

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### What's new?

- The N-terminus of glutamate decarboxylase (GAD<sub>65</sub>) contributes little to epitopes recognized by Type 1 diabetes associated GAD antibodies.
- Radioimmunoassays using N-terminally truncated  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585) improve the specificity of GAD antibody measurement.
- We show that using a more radically truncated  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) radiolabel to measure GAD antibodies does not impact assay sensitivity and improves diabetes specificity compared with full-length  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585).

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- Relatives of people with Type 1 diabetes who were GAD antibody-positive using truncated radiolabels had increased risk of progression to diabetes within 15 years compared with those positive for GAD(1–585) antibody alone, and were at similar risk to those found positive using a high-performing commercial GAD antibody enzyme-linked immunosorbent assay.

## Abstract

**Aims** Glutamate decarboxylase (GAD) antibodies are the most widely used predictive marker for Type 1 diabetes, but many individuals currently found to be GAD antibody-positive are unlikely to develop diabetes. We have shown previously that radioimmunoassays using N-terminally truncated  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585) offer better disease specificity with similar sensitivity to full-length  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585). To determine whether assay performance could be improved further, we evaluated a more radically truncated  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) radiolabel.

**Methods** Samples from people with recent-onset Type 1 diabetes ( $n = 157$ ) and their first-degree relatives ( $n = 746$ ) from the Bart's–Oxford family study of childhood diabetes were measured for GAD antibodies using  $^{35}\text{S}$ -labelled GAD<sub>65</sub>(143–585). These were screened previously using a local radioimmunoassay with  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585). A subset was also tested by enzyme-linked immunosorbent assay (ELISA), which performs well in international workshops, but requires 10 times more serum. Results were compared with GAD antibody measurements using  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585) and  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585).

**Results** Sensitivity of GAD antibody measurement was maintained using  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) compared with  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585) and  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585). Specificity for Type 1 diabetes was improved compared with  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585), but was similar to  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585). Relatives found to be GAD antibody-positive using these truncated labels were at increased risk of diabetes progression within 15 years, compared with those positive for GAD(1–585) antibody only, and at similar risk to those found GAD antibody-positive by ELISA.

**Conclusions** The first 142 amino acids of GAD<sub>65</sub> do not contribute to epitopes recognized by Type 1 diabetes-associated GAD antibodies. Low-volume radioimmunoassays using N-terminally truncated  $^{35}\text{S}$ -GAD<sub>65</sub> are more specific than those using full-length GAD<sub>65</sub> and offer practical alternatives to the GAD antibody ELISA for identifying children at increased risk of Type 1 diabetes.

## <H1>Introduction

Approximately 80% of children diagnosed with Type 1 diabetes have glutamate decarboxylase antibodies [1]. This marker is used widely, both clinically and in research, to identify individuals at increased risk of Type 1 diabetes, assisting in diabetes diagnosis and as an inclusion criterion for therapeutic intervention trials [2].

Radioimmunoassays are traditionally used to measure GAD antibodies, although other methods including enzyme-linked immunosorbent assay (ELISA) [3], electrochemiluminescence [4] or luciferase immunoprecipitation system assays [5] are becoming increasingly popular. Despite well-established harmonization programmes [6] and international proficiency workshops to standardize and improve GAD antibody measurement [7], many individuals testing positive for this antibody do not progress to diabetes [8]. A commercial bridging ELISA, although requiring more serum per test than radioimmunoassays (50 µl vs. 4 µl, respectively), consistently showed higher specificity in international workshops [9]. This improved specificity may be explained in part by reduced access of autoantibodies to the N-terminus of GAD<sub>65</sub> in the ELISA configuration. The primary antigenic region of Type 1 diabetes-associated GAD antibody is found in the middle and C-terminus of GAD<sub>65</sub>; antibodies recognizing only the N-terminal region were not associated with progression to multiple islet autoimmunity or diabetes [10,11]. We therefore developed an N-terminally truncated GAD<sub>65</sub> radiolabel, <sup>35</sup>S-GAD<sub>65</sub>(96–585), for use as tracer. Removing the first 95 amino acids, comprising the first three exons of GAD<sub>65</sub> [12,13] increased the specificity of GAD antibody radioimmunoassays without loss of sensitivity, improving their workshop performance [11]. We also demonstrated that autoantibodies measured with this construct, in first-degree relatives (FDR) of people with Type 1 diabetes, were more closely associated with diabetes risk [14]. Fewer than half of FDRs found positive for GAD<sub>65</sub>(96–585) antibodies by radioimmunoassay, however, were likely to develop diabetes within 25 years.

To discover whether further N-terminal truncation could improve the specificity of GAD antibody measurement by radioimmunoassay, we evaluated several GAD<sub>65</sub> constructs in which regions up to and including exon 6 were deleted (Fig. 1a,b). We also investigated how GAD antibody levels measured by radioimmunoassay with truncated GAD<sub>65</sub> labels or by ELISA compared, and examined how positivity by each method contributed to the overall risk of developing diabetes.

## <H1>Methods

### <H2>Population

#### <H3>*Bart's–Oxford family study*

Sera were available from the Bart's–Oxford (BOX) family study. This population-based study was established in 1985 [15] and has recruited more than 3000 children with Type 1 diabetes diagnosed before the age of 21 years in the Oxford region of the UK, 76% of whom are still followed up. Today,

over 6000 FDRs of the probands have been recruited and followed prospectively for disease development by annual questionnaire. All families were tested for autoantibodies to insulin, full-length GAD<sub>65</sub> and islet antigen 2, using established local assays [16]. Those found to be antibody-positive were also tested for autoantibodies to zinc transporter 8 (ZnT8) [17].

### *<H3>Cohort for initial screening of the GAD<sub>65</sub> constructs*

Initial evaluation of the sensitivity and specificity of the N-terminally truncated <sup>35</sup>S-GAD<sub>65</sub> constructs was performed using a panel of sera from 11 people with Type 1 diabetes and 25 BOX relatives previously found to be GAD<sub>65</sub>(96–585) antibody-positive [14]. These relatives were considered at low-risk of diabetes because, although GAD<sub>65</sub>(96–585) antibody-positive, they did not have any additional islet autoantibodies and had not progressed to diabetes during follow-up. The constructs tested with this panel included: GAD<sub>65</sub>(115–585), GAD<sub>65</sub>(143–585), GAD<sub>65</sub>(160–585), GAD<sub>65</sub>(188–585) and GAD<sub>65</sub>(242–585) (Fig. 1c).

### *<H3>Cohorts for evaluating the performance of GAD(143–585) antibody*

Sera with sufficient volume from 157 people (94 male; median age 11.7 years, age range 1.3–20.9 years) with recent-onset Type 1 diabetes (median duration 1 day, range –7 to 90 days) were selected at random from 613 participants [14] to determine the disease sensitivity of GAD antibody measurement using the N-terminally truncated <sup>35</sup>S-GAD<sub>65</sub> constructs (Table 1, Fig. 2a).

To evaluate discrimination of risk of diabetes progression, sera were available from 282 (138 male; median age 31.4 years, range 1.3–57.4 years) of 283 FDRs previously found to be GAD antibody-positive with the local radioimmunoassay (Table 1, Fig. 2b).

Assay specificity was further evaluated by testing the first available sample from 463 FDRs (229 male; median age 33.3 years, age range 1.4–57.3 years) who previously tested GAD<sub>65</sub>(1–585) antibody negative with the local radioimmunoassay. Samples were selected at random from 4187 GAD antibody-negative individuals but were enriched with 33 of 179 individuals who developed diabetes during follow-up (Table 1, Fig. 2b) [14].

### *<H3>Cohorts for evaluating the performance of the GAD antibody ELISA*

Limited serum availability prevented ELISA testing of all samples, such that 81 of the 157 people with Type 1 diabetes (52%), 258 of the 282 GAD antibody-positive relatives (91%) and 418 of the 463 GAD antibody-negative relatives (90%) were tested for GAD antibodies by ELISA (Table 1; Figs S1 and S2).

Results obtained with  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) and ELISA were compared with previous GAD(1–585) and GAD(96–585) antibody measurements [14,16].

## <H2>Autoantibody assays

### <H3>Radioimmunoassay

Sera (2  $\mu\text{l}$ , in duplicate) screened using the local GAD antibody assay [16] were remeasured using the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) harmonized GAD antibody radioimmunoassay protocol [6]. The GAD<sub>65</sub> antigens were encoded in the PCMVNTNT plasmid vector (Promega, Madison, WI, USA).  $^{35}\text{S}$ -Methionine-labelled GAD<sub>65</sub> tracers were synthesized using the TnT quick-coupled *in vitro* transcription/translation system (Promega). Results were converted to Diabetes and Kidney (DK) units/ml using a standard curve. In the 2017 Islet Autoantibody Standardization Program (IASP) workshop, the adjusted sensitivity at 95% specificity for GAD(1–585), GAD(96–585) and GAD(143–585) antibody assays was 74%, 80% and 82%, respectively.

### <H3>ELISA

Sera (25  $\mu\text{l}$  in duplicate) were measured for GAD antibodies by ELISA (RSR Ltd, Cardiff, UK), following the manufacturer's instructions. In brief, divalent GAD antibodies in serum form bridges between GAD<sub>65</sub> immobilized on the ELISA plate wells and biotinylated GAD<sub>65</sub> in solution [3,16]. Quantification of bound GAD antibodies was achieved following addition of streptavidin peroxidase and the substrate 3,3',5,5'-tetramethylbenzidine. Stop solution was added and the absorbance of the reaction mixture at 405 nm was read. Results were converted to NIBSC 97/550 units using the calibrators provided.

### <H3>Antibody thresholds

Thresholds for the harmonized GAD antibody radioimmunoassays that used the three GAD<sub>65</sub> constructs investigated in detail were set at the 97.5th percentile of 221 healthy schoolchildren (Fig. 2a) [18], equal to 13.5, 12.8 and 11.8 DK units/ml for  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585),  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585) and  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585), respectively.

Because of limited serum availability, the threshold for the GAD antibody ELISA was set at the 97.5th percentile of 180 of the 221 healthy schoolchildren, equal to 6.0 NIBSC 97/550 units (Fig. S1). The equivalent thresholds were 12.9, 14.0 and 11.8 DK units/ml for the same samples when measured with  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585),  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585) and  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585), respectively.

## <H2>Statistical analysis

Wilcoxon matched-pairs signed-ranked test was used to compare signal-to-noise ratios (SNRs) and McNemar's test with Yate's correction for comparing antibody status between constructs and assay formats. The Kaplan–Meier test was used for survival analysis and the Mantel–Cox log-rank test for comparing survival between groups. For all analyses, a two-tailed *P*-value of < 0.05 was considered significant. The partial area (90th percentile) under the curve (pAUC) of the receiver operating characteristic (ROC) with 95% confidence intervals (CI) was calculated assuming a nonparametric distribution of results using R software, version 3.2.2. Other statistical analyses were performed using GraphPad Prism version 6.

## <H1>Results

### <H2>Initial construct screening

N-terminally truncated <sup>35</sup>S-GAD<sub>65</sub> constructs were screened in the initial evaluation (Fig. 1) to discover which were likely to offer the best discrimination between diabetes and non-progression. A SNR (*sample cpm ÷ negative standard cpm*) was calculated using a negative standard included in all assays. The SNR of <sup>35</sup>S-GAD<sub>65</sub>(143–585) in people with Type 1 diabetes was most comparable with that with <sup>35</sup>S-GAD<sub>65</sub>(96–585). Furthermore, this construct had low background binding and gave a lower SNR in the low-risk relatives compared with <sup>35</sup>S-GAD<sub>65</sub>(96–585), suggesting that <sup>35</sup>S-GAD<sub>65</sub>(143–585) had the potential to improve GAD antibody specificity without reducing sensitivity. <sup>35</sup>S-GAD<sub>65</sub>(143–585) was, therefore, selected for detailed evaluation (Fig. 1d, Table S1).

### <H2>Sensitivity was maintained with GAD(143–585) antibody in people with Type 1 diabetes and high-risk GAD antibody-positive relatives

Of 157 people with Type 1 diabetes, 127 (80%) were positive for GAD(1–585) antibodies, 127 (80%) for GAD(96–585) antibodies and 128 (82%) for GAD(143–585) antibodies, with 124 (79%) positive for all three specificities (Fig. 2a). Using ROC analysis, pAUC values were 0.077 (95% CI 0.071–0.083), 0.076 (95% CI 0.069–0.081) and 0.077 (95% CI 0.071–0.082), for GAD(1–585), GAD(96–585) and GAD(143–585) antibodies, respectively (Fig. S3a). Sensitivity at 95% specificity (AS95) was 84% for all three constructs.

Sensitivity was also similar in the 109 relatives who progressed to diabetes during follow-up and/or had autoantibodies to additional islet antigens (Table S2), with 102 (94%) positive for GAD(1–585) antibodies, 98 (90%) for GAD(96–585) antibodies and 100 (92%) for GAD(143–585) antibodies. Ninety-five (87%) of these high-risk relatives were positive for all three specificities.



## **<H2>Fewer GAD(1–585) antibody-positive relatives who had no additional islet autoantibodies and had not progressed to diabetes tested positive using truncated <sup>35</sup>S-GAD<sub>65</sub> constructs**

Of the 173 low-risk relatives, 156 (90%) were positive for GAD(1–585) antibodies compared with 108 (62%) for GAD(96–585) antibodies ( $P < 0.0001$ ) and 98 (57%) for GAD(143–585) antibodies ( $P < 0.0001$ ). The proportion of these low-risk relatives found positive for GAD(143–585) antibodies was reduced compared with those positive for GAD(96–585) antibodies ( $P = 0.02$ ) (Table S3).

## **<H2>Truncated <sup>35</sup>S-GAD<sub>65</sub> constructs improved discrimination of diabetes risk**

In 258 relatives who tested GAD antibody-positive using the harmonized radioimmunoassay with <sup>35</sup>S-GAD<sub>65</sub>(1–585), the 15-year risk of diabetes was 26% (95% CI 20–32%). Within this group, positivity for GAD(96–585) and GAD(143–585) antibodies showed a similar ability to further stratify diabetes risk ( $P < 0.0001$ , for both). Nevertheless, positivity for GAD(143–585) antibodies did not improve discrimination of risk compared with GAD(96–585) antibodies. Individuals positive for GAD(96–585) antibodies had a 33% (95% CI 26–41%) risk of developing diabetes within 15 years, whereas individuals positive for GAD(143–585) antibodies had a 34% (95% CI 27–42%) risk (Fig. 3a).

## **<H2>Sensitivities of the ELISA and radioimmunoassays in people with Type 1 diabetes and high-risk individuals were similar**

The prevalence of GAD antibodies in the 81 people with Type 1 diabetes measured by ELISA (86%) was similar to that in the same individuals measured by radioimmunoassay (80% for all three constructs,  $P > 0.05$  for all comparisons) (Table S4 and Fig S1). ROC analysis of these 81 people with Type 1 diabetes and 180 healthy schoolchildren, found the pAUC for GAD antibodies measured by ELISA was 0.084 (95% CI 0.076–0.091), compared with 0.077 for GAD(1–585) antibodies (95% CI 0.069–0.085), 0.076 for GAD(96–585) antibodies (95% CI 0.066–0.085) and 0.077 for GAD(143–585) antibodies (95% CI 0.069–0.084) when measured by radioimmunoassay ( $P > 0.05$  for all comparisons) (Fig. S3b). AS95 was 84%, 86%, 85% and 89% for GAD(1–585), GAD(96–585) and GAD(143–585) antibodies and the ELISA, respectively.

Of 85 relatives who progressed to diabetes and/or had autoantibodies to additional islet antigens, 74 (87%) were found GAD antibody-positive by ELISA compared with 78 (92%,  $P > 0.05$ ), 71 (84%,  $P > 0.05$ ) and 76 (89%,  $P > 0.05$ ) found positive for GAD(1–585), GAD(96–585) and GAD(143–585) antibodies by radioimmunoassay, respectively (Table S4).



## **<H2>Fewer GAD(1–585) antibody-positive relatives who had no additional islet autoantibodies and had not progressed to diabetes tested positive using the ELISA**

Of the 173 low-risk GAD antibody-positive relatives, 77 (45%) retested positive by ELISA compared with 156 (90%,  $P < 0.0001$ ) using  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585), 105 (61%,  $P < 0.0001$ ) using  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585) and 98 (57%,  $P = 0.0001$ ) using  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) (Table S5).

## **<H2>GAD antibody measurement using ELISA did not improve discrimination of risk compared with truncated $^{35}\text{S}$ -GAD<sub>65</sub> constructs**

In the 234 relatives positive for GAD(1–585) antibody tested for GAD antibodies by ELISA, risk of developing diabetes within 15 years was 21% (95% CI 16–28%;  $P < 0.0001$ ). Within this group, positivity for GAD(96–585) and GAD(143–585) antibodies, as well as GAD antibodies measured by ELISA, further stratified risk of diabetes ( $P < 0.0001$ , for all comparisons). However, 15-year risk in these individuals was similar when measured by ELISA (31%, 95% CI 24–41%) or by radioimmunoassays using  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585) (28%, 95% CI 21–36%) or  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) (29%, 95% CI 22–38%) (Fig. 3b).

## **<H2>Relatives who previously screened GAD antibody negative**

Of 463 relatives who previously tested negative for GAD antibodies using the local radioimmunoassay, 12 (3%) retested positive for GAD(1–585) antibodies, seven (2%) for GAD(96–585) antibodies and 13 (3%) for GAD(143–585) antibodies ( $P > 0.05$  for all comparisons). Three samples were positive with all three truncated constructs (Fig. 2b). Within this subset, 33 relatives had progressed to diabetes. Of these, none were positive for GAD(1–585) antibodies, one (3%) was positive for GAD(96–585) antibodies and two (6%) for GAD(143–585) antibodies (Tables S2 and S3).

Similar results were seen for the 389 GAD antibody-negative relatives who did not progress to diabetes during follow-up, as 11 (3%) were positive using the ELISA compared with eight (2%,  $P > 0.05$ ) using  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585), three (1%,  $P = 0.04$ ) with  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585) and nine (2%,  $P > 0.05$ ) with  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) (Table S5). Of the relatives who progressed, 29 were tested for GAD antibodies using ELISA; one (3%) of these was positive with ELISA, whereas a different sample (3%) was positive using  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) (Table S4).

## **<H1>Discussion**

A similar proportion of people with Type 1 diabetes and relatives who progressed to diabetes were found positive for GAD antibodies with radioimmunoassays using  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585) and  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) or full-length  $^{35}\text{S}$ -GAD<sub>65</sub>, indicating that sensitivity was maintained using the N-terminally truncated GAD<sub>65</sub> constructs. Furthermore, relatives positive for GAD antibodies using these truncated labels were at increased risk of progression to diabetes within 15 years compared

with those who retested positive for GAD(1–585) antibodies using the harmonized assay, and at similar risk to those found GAD antibody-positive using the commercial ELISA.

This study benefits from the inclusion of many participants selected from a well-characterized, population-based family study with common assay thresholds set using a schoolchild cohort from the same geographical area. Although the relatives analysed were not followed from birth, and, therefore, may not be perfectly representative of the general population, they nonetheless provide a unique opportunity to study the relationship between the autoantibody response to GAD<sub>65</sub> and diabetes development over a prolonged follow-up, extending up to 30 years. In addition, all samples from people with Type 1 diabetes were collected within 3 months of diagnosis, minimizing the likelihood of negative seroconversion. Furthermore, antibodies to the <sup>35</sup>S-GAD<sub>65</sub> constructs have been measured using standardized and validated radioimmunoassays, whose performance was assessed in international workshops [6,9,11]. Limitations of our study were that all samples were pre-screened using a local full-length GAD autoantibody radioimmunoassay and we were unable to test all samples by ELISA, because of inadequate serum volumes. A large cohort of GAD antibody-negative relatives was included to address any resulting selection bias and help evaluate the performance of the different assays when used for primary screening. Overall, these attributes allowed a detailed evaluation of disease specificity and sensitivity.

Studies using engineered GAD<sub>65</sub> proteins or monoclonal antibodies demonstrated that GAD antibodies associated with Type 1 diabetes primarily target epitopes found in the middle and C-terminal domains of GAD<sub>65</sub> [19–25]. Using GAD67/65 chimeras, the BABYDIAB study identified the primary GAD antibody epitope within GAD<sub>65</sub> residues 96–444, with epitope spreading to the N-terminal region being common [26]. Another group using competition radioimmunoassays with recombinant Fabs of four GAD<sub>65</sub>-specific monoclonal antibodies, found that epitope spreading from the C-terminus (amino acids 483–585) and middle region (amino acids 195–365) to the N-terminus (amino acids 96–173) was associated with progression, while epitope reactivity was stable in low-risk individuals [27]. This approach was also used to characterize GAD antibody epitopes in a small selection of people with Type 1 diabetes or latent autoimmune diabetes of adults, FDRs and healthy individuals. Competition with a Fab recognizing amino acids 96–173 (DP-D) reduced median binding by 80%. In people with Type 1 diabetes, 3% were fully inhibited by DP-D and 62% partially, implicating these amino acids as contributors to a disease-specific epitope. Although this inhibition may be related to amino acids 143–173, the discrepancy with our findings could be explained by effects of Fab binding on other regions of the molecule. Of note, GAD antibody-positive samples inhibited by a Fab recognizing amino acids 308–365 also showed competition by DP-D ( $P = 0.006$ ) [24].

We investigated a range of truncated constructs including GAD<sub>65</sub>(115–585), GAD<sub>65</sub>(143–585), GAD<sub>65</sub>(160–585), GAD<sub>65</sub>(188–585) and GAD<sub>65</sub>(242–585). Removal of amino acids up to position 188 had only a small impact on binding in people with Type 1 diabetes compared with <sup>35</sup>S-GAD<sub>65</sub>(96–585), whereas truncation at position 242, comprising the first 6 exons, severely reduced binding in

sera from people with Type 1 diabetes. These findings suggest that truncations up to position 188 are unlikely to disrupt folding of the major GAD antibody epitopes but confirm that residues 188–242 are probably critically important to the integrity of diabetes-relevant epitopes [24,27]. Although antibody binding was reduced in low-risk samples using  $^{35}\text{S}$ -GAD<sub>65</sub>(160–585) and  $^{35}\text{S}$ -GAD<sub>65</sub>(188–585), critically, the poor SNRs seen with these constructs resulted from increased background binding. Consequently,  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) was selected for detailed evaluation. Overall, a small improvement in disease specificity, without a loss of sensitivity was seen with this construct when compared with  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585), indicating that the first 142 amino acids do not make an important contribution to Type 1 diabetes-associated GAD antibody epitopes. Assays using more radically truncated GAD<sub>65</sub> antigens could, therefore, offer alternatives to GAD<sub>65</sub>(96–585) for GAD antibody measurement.

The commercial GAD antibody ELISA performed well, but did not offer significant improvements in discriminating risk of developing diabetes within 15 years, compared with radioimmunoassays using the truncated  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585) or  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) constructs. Improved specificity may be expected when re-assaying samples using a different method, as non-specific binding is less likely to be reproduced in a different assay format. However, we found little evidence that the ELISA would identify additional progressors who screened negative by radioimmunoassay. Furthermore, the ELISA requires 50 µl serum per test, compared with only 4 µl for the radioimmunoassay. This is an important consideration, as GAD antibody seroconversion often occurs in early infancy, and therefore early childhood is considered the optimum age for population screening for Type 1 diabetes. Capillary blood samples, which can facilitate population screening and recruitment to trials, typically yield < 100 µl serum [28,29]. Low-volume radioimmunoassays with a proven track record therefore provide a useful tool for Type 1 diabetes prediction. The flexibility of this method enables detailed epitope characterization using truncated GAD<sub>65</sub> antigens and chimeras, which may help to further delineate the autoimmune response. Radioimmunoassay can also evaluate other autoantibody features, such as affinity, which may further discriminate risk in GAD antibody-positive individuals [10,30].

We have shown that individuals who are positive for GAD antibodies measured using truncated  $^{35}\text{S}$ -labelled GAD<sub>65</sub> constructs have a higher risk of developing diabetes compared with those positive for full-length GAD antibodies. Thus, positivity for GAD(96–585) or GAD(143–585) antibodies may be a useful inclusion criterion for therapeutic intervention trials, which target high-risk individuals.

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## Competing interests

None declared.

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## Author contributions

AJKW, RCW, PA, LP and VL initiated the study. RCW, AJKW, CB, DL, SLG, BTG, IM, DS, KAC and VL researched the data. RCW and AJKW wrote the manuscript. RCW, AJKW, KMG, AEL, VL, PA and LP contributed to the discussion. All authors revised and reviewed the manuscript. AJKW is responsible for the integrity of this work.

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**FIGURE 1.** (a) Schematic of the N-terminally truncated GAD<sub>65</sub> constructs screened for improved radioimmunoassay specificity. (b) Exon map of the N-terminal region of GAD<sub>65</sub> (from Matsukawa and Ueno [13]). (c) Schematic of the experimental design for the screening process. The initial evaluation panel was used to select the most promising truncated GAD construct for more detailed comparison with GAD<sub>65</sub>(96–585). Samples with a range of GAD<sub>65</sub>(96–585) antibody titres were selected (median in people with diabetes: 421, range 159–1447 DK units/ml; median in relatives: 42, range 18–369 DK units/ml). (d) Signal-to-noise ratios (SNR) from 11 people with recent-onset Type 1 diabetes (▲) and 25 single GAD(96–585) antibody-positive relatives who had not developed diabetes during follow-up (○), measured for GAD antibodies using <sup>35</sup>S-



GAD<sub>65</sub>(96–585), <sup>35</sup>S-GAD<sub>65</sub>(115–585), <sup>35</sup>S-GAD<sub>65</sub>(143–585), <sup>35</sup>S-GAD<sub>65</sub>(160–585), <sup>35</sup>S-GAD<sub>65</sub>(188–585) and <sup>35</sup>S-GAD<sub>65</sub>(242–585) by radioimmunoassay. The SNR was calculated using a negative standard included in all assays (*sample cpm ÷ negative standard cpm*). Bars represent the median and interquartile range of values. Differences in the SNRs of people with Type 1 diabetes compared with relatives who were diabetes-free shows discrimination of diabetes and non-progression.

**FIGURE 2.** Selection scheme of samples tested for GAD antibodies using radioimmunoassay or ELISA, showing radioimmunoassay results for (a) healthy schoolchildren and people with recent-onset Type 1 diabetes or (b) BOX relatives. Samples were re-assayed with the NIDDK harmonized radioimmunoassay protocol using <sup>35</sup>S-GAD<sub>65</sub>(1–585), <sup>35</sup>S-GAD<sub>65</sub>(96–585) and <sup>35</sup>S-GAD<sub>65</sub>(143–585), and a proportion assayed using ELISA. Similar numbers of patients were found to be positive for GAD antibodies using all three radiolabelled constructs. Fewer relatives who were found previously to be GAD antibody-positive using a local assay were positive on re-assay for GAD(96–585) or GAD(143–585) antibodies compared with GAD(1–585) antibodies ( $P < 0.0001$ , for all).

**FIGURE 3.** (a) Kaplan–Meier survival curve for 258 first-degree relatives found positive for GAD(1–585) antibodies in the harmonized assay, according to positivity for GAD(96–585) and GAD(143–585) antibodies. GAD(96–585) and GAD(143–585) antibodies identified relatives at increased risk of diabetes progression. Individuals positive for GAD(96–585) antibodies had a 33% (95% CI 26–41%) risk of developing diabetes within 15 years, whereas those positive for GAD(143–585) antibodies had a 34% (95% CI 27–42%) risk. Few relatives who were positive for GAD(1–585) antibodies, but negative for GAD(96–585) or GAD(143–585) antibodies developed diabetes within 30 years of follow-up. (b) Kaplan–Meier survival curve for 234 first-degree relatives positive for GAD(1–585) antibodies according to positivity for GAD(96–585) and GAD(143–585) antibodies measured by radioimmunoassay and GAD antibodies measured by ELISA. These relatives are a subset of the cohort analysed in (a). Measurement of GAD antibodies using <sup>35</sup>S-GAD<sub>65</sub>(96–585), <sup>35</sup>S-GAD<sub>65</sub>(143–585) and the ELISA method identified relatives at increased risk of diabetes progression ( $P < 0.0001$ , respectively). Individuals positive for GAD(96–585) antibodies had a 28% (95% CI 21–36%) risk of developing diabetes within 15 years, whereas those positive for GAD(143–585) antibodies had a 29% (95% CI 22–38%) risk and individuals found positive using ELISA had a 31% (95% CI 24–41%) risk. Few relatives who were positive for GAD(1–585) antibodies but negative for GAD(96–585) and GAD(143–585) antibodies or by ELISA developed diabetes within 30 years of follow-up.

## <H1>Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Author to supply caption.

**Figure S2.** Author to supply caption.

**Figure S3.** Author to supply caption.

**Table S1.** Author to supply table title.

**Table S2.** GAD antibody levels in high-risk BOX samples measured with  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585),  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585) and  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585).

**Table S3.** GAD antibody levels in low-risk BOX samples measured with  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585),  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585) and  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585).

**Table S4.** GAD antibody levels in high-risk BOX samples measured with  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585),  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585) and  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) in RIA and GAD<sub>65</sub>(1–585) in ELISA.

**Table S5.** GAD antibody levels in low-risk BOX samples measured with  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585),  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585) and  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) in RIA and GAD<sub>65</sub>(1–585) in ELISA.

**Table 1** Characteristics of people with Type 1 diabetes and first-degree relatives participating in the BOX family study whose samples were originally screened for glutamic acid decarboxylase (GAD) antibodies using a local radioimmunoassay and were retested using  $^{35}\text{S}$ -GAD<sub>65</sub>(1–585),  $^{35}\text{S}$ -GAD<sub>65</sub>(96–585) and  $^{35}\text{S}$ -GAD<sub>65</sub>(143–585) by harmonized radioimmunoassay and GAD<sub>65</sub>(1–585) using an ELISA

	People with Type 1 diabetes ( <i>n</i> = 157)	GAD antibody-positive relatives ( <i>n</i> = 282)		GAD antibody-negative relatives ( <i>n</i> = 463)	
		Progressor ( <i>n</i> = 72)	Non-progressor ( <i>n</i> = 210)	Progressor ( <i>n</i> = 33)	Non-progressor ( <i>n</i> = 430)
No. of males (%)	94 (60)	36 (50)	102 (49)	24 (73)	205 (48)
Median age, years (range)	11.7 (1.3–20.9)	32.5 (1.6–52.9)	30.9 (1.33–57.4)	39.1 (1.4–56.2)	32.6 (1.7–57.3)
Median age at diagnosis, years (range)	11.8 (1.3–20.9)	38.9 (3.2–69.8)	–	52.0 (3.3–68.5)	–
Median follow-up, years	1	7.4	17.6	12.4	7.6
Median diabetes duration, days (range)	–7 to 90	0.2–27.8	0.6–30.8	1.2–23.8	0–31.0
Additional autoantibodies*	142	41	37	5	11‡
Islet antigen 2 antibodies	122	24	13	2	1
Indole 3 acetic acid	71†	23	23	4	10
ZnT8A	113	30	17	1	–
No. tested for GAD antibodies using ELISA (%)	81 (52)	53 (74)	205 (98)	29 (88)	389 (90)

ELISA, enzyme-linked immunosorbent assay (RSR Ltd, Cardiff, UK).

\*Additional autoantibodies are from islet antigen 2, indole 3 acetic acid and zinc transporter type 8.

†57 not tested for indole 3 acetic acid as the sample was taken more than two weeks after diagnosis of Type 1 diabetes and any antibodies measured may be to endogenous insulin induced by insulin therapy.

‡Not tested for zinc transporter type 8.

Figure 1a

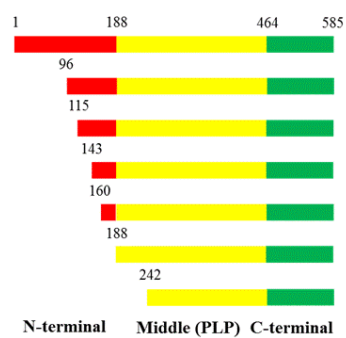


Figure 1b

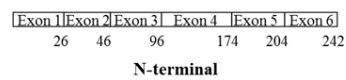


Figure 1c

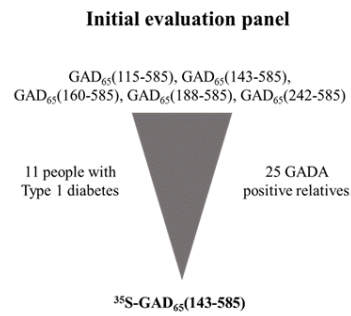


Figure 1d

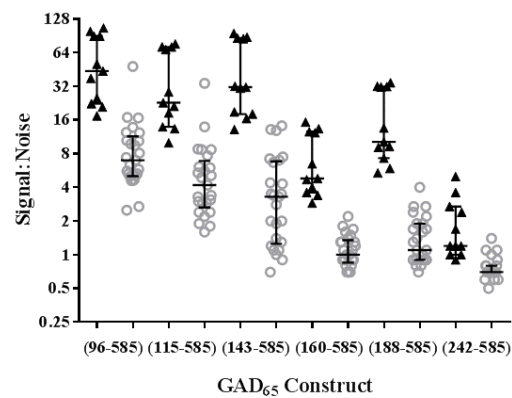


Figure 2a

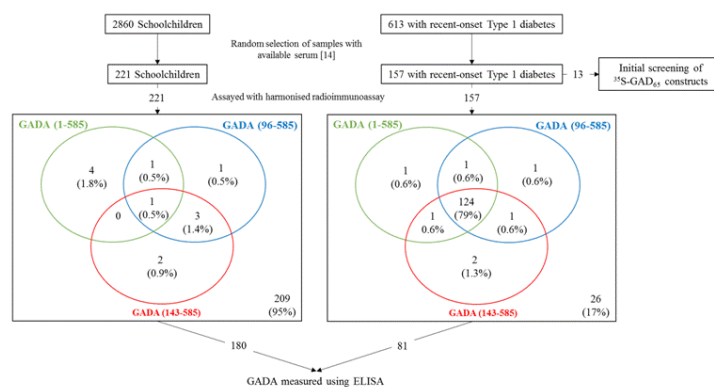


Figure 2b

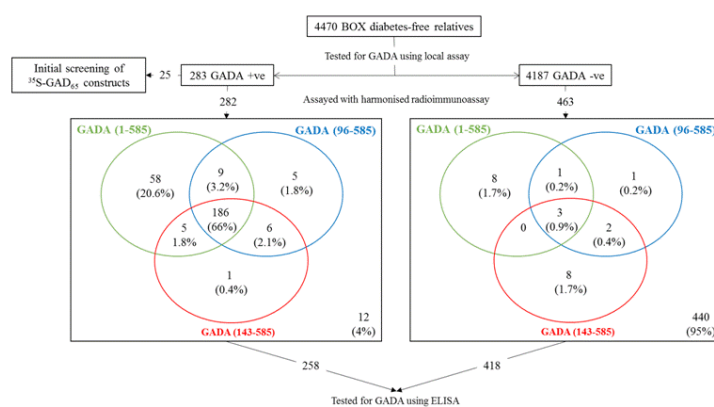


Figure 3a

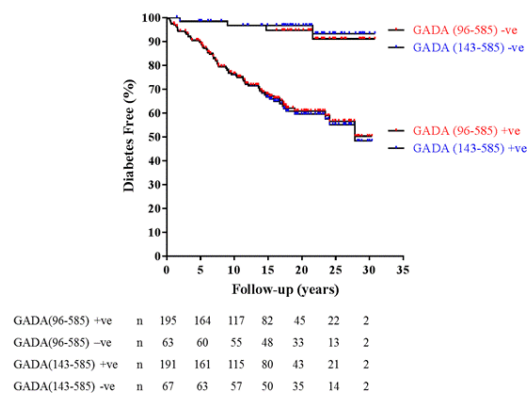


Figure 3b

